BAL Fluid Contains Detectable Superoxide Dismutase 1 Activity*

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Objective: **This study determined which, if any, of the three superoxide dismutase (SOD) enzyme activities were detectable in BAL fluid (BALF).**
Background: **The identity and concentrations of antioxidant molecules in BALF have not been fully characterized. One important class of antioxidants is that of the SOD enzymes.**
Methods: **BALF from control nonsmokers (n=9), smokers (n=7), and asthmatic subjects (n=12) were examined for SOD activity by a modified pyrogallol auto-oxidation method. The particular SOD enzyme or enzymes responsible for any activity were identified based on activity inhibition data and gel filtration column chromatography patterns.**
Results: **SOD activity was detected in all samples. Unlike serum or some other fluids, in which the enzyme extracellular SOD accounts for virtually all SOD activity, the enzyme SOD 1 accounted for virtually all SOD activity. BALF SOD activities were lower for smokers than for control or asthmatic subjects ($p<0.05$).**
Conclusion: **BALF SOD 1 activities can be measured as part of lung antioxidant studies. Data from a limited number of subjects suggested that smokers can have low BALF SOD values.**
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Key words: antioxidants; asthma; bronchoalveolar lavage fluid; smokers; superoxide dismutase

Abbreviations: BALF=BAL fluid; EC=extracellular; SOD=superoxide dismutase

Many lung diseases are thought to involve oxidative actions of free radicals such as superoxide.[1-3] Therefore, resistance to such diseases should be affected by the balance between radical formation and elimination. Elimination is fostered by enzymatic and nonenzymatic antioxidant molecules. The most accessible means of studying lung antioxidants is bronchoscopy to obtain samples such as BAL fluid (BALF). However, the range of antioxidants detectable in BALF is not fully characterized.

One group of antioxidant molecules, the superoxide dismutase (SOD) enzymes, eliminate superoxide radicals.[1-6] Mammals possess three distinct SOD enzymes.[1-6] One occurs in cytosol, lysosomes, and peroxisomes and is called cytosolic SOD, SOD 1, or copper-zinc SOD. A second is mitochondrial SOD, also called SOD 2 or manganese SOD. The third enzyme, a copper-zinc protein, is termed extracellular (EC) SOD. EC SOD activity has been detected in tissues and some body fluids.[4-6] The present study determined which, if any, of the three SOD enzymes could be measured in BALF from smokers, nonsmokers, and asthmatic patients.

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Materials and Methods

Bronchoscopy to collect BALF samples was performed at the Ohio State University Hospital and the University of Wisconsin Medical School. Male and female subjects, aged 20 to 42 years, were smokers with no pulmonary disorders, healthy nonsmokers, or asthmatic subjects. Smokers had a mean pack-year history of 19.7±3.5. Asthma was defined as a history of recurrent wheezing that responded to inhaled β-agonists. In addition, subjects for this study demonstrated increased airway hyperresponsiveness and allergic responses to common skin aeroallergens (5±3 positive skin tests). Asthmatic subjects had a mean FEV_1% of 84±4 and an FEV_1/FVC ratio of 74±2. Bronchoscopy was done during periods of clinical stability (no attacks or therapy changes for 1 month). When applicable, β-agonist therapy was discontinued 8 h before bronchoscopy; theophylline, 24 to 48 h before; and steroids and cromolyn sodium, 1 mo before.

BALF sample collection procedures have been described previously.[7] After collection, samples were centrifuged at 400 g for 10 min to remove cells. Most samples were stored frozen at −80°C before concentration and analysis. As noted in the "Results" section, three new individual samples were concen-

trated and analyzed on the day of sample collection. These samples were used to compare gel filtration results for freshly obtained and frozen samples.

Except for gel filtration work, samples were concentrated on Minicon B15 devices (Amicon; Beverly, Mass). Total protein was measured by the Bio-Rad protein reagent and procedure (Bio-Rad Laboratories; Mellville, NY). SOD activities were assayed by a modified pyrogallol auto-oxidation method[8] with the use of conditions and units described earlier.[9] Cyanide and ethanol-chloroform inhibition of activity were done as described previously.[8,9]

The sensitivity of BALF SOD activity to antiSOD 1 was determined with commercial antibody (The Binding Site; San Diego, Calif). Initially, SOD activity was determined for the BALF samples under consideration. Next, an equivalent activity of purified human SOD 1 (Calbiochem-Novabiochem Corporation; San Diego, Calif) was mixed with various amounts of antiSOD 1 to determine how much antibody gave 100% inhibition of the SOD activity. Then, twice this amount of antibody was incubated with the BALF sample for 90 min at 37°C. As a control, sample aliquots were incubated with phosphate-buffered saline for the same time. Following this incubation, control samples or the samples plus antibody were incubated with protein G-agarose (Boehringer Mannheim; Indianapolis) for 25 min at 37°C. The protein G-agarose was then separated from the solutions by microcentrifugation, and the supernatants were assayed for any remaining SOD activity.

For gel filtration analysis, BALF samples were concentrated by ultrafiltration using concentrators (Centricon-10; Amicon). Then, column chromatography was done with Sephadex G-150 (Sigma Chemical; St. Louis) using conditions cited in Figure 1. Previous to BALF analysis, human ceruloplasmin (Sigma) and bovine liver SOD 1 (DDI; Mountainview, Calif) were chromatographed as molecular weight markers. Ceruloplasmin was detected by its blue color (absorbance at 610 nm) and SOD 1, by its enzyme activity.

Levels of SOD activity for asthmatic subjects, smokers, and control subjects were compared by one-way analysis of variance plus least significant differences analysis (ANOVA + LSD). Significance was set at $p<0.05$.

Results

SOD activity was detectable in all BALF samples tested (9 nonsmokers, 7 smokers, and 12 asthmatic subjects). As shown in Table 1, SOD activities for smokers were significantly lower than those for control or asthmatic subjects. Asthmatic subjects showed a trend toward higher SOD values than control subjects, but there was no statistically significant difference between the groups (ANOVA + LSD, $p>0.05$).

Further analysis of SOD activity of 5 samples (3 smokers, 2 control subjects) showed about 90% inhibition by cyanide, but no inhibition by ethanol-chloroform. This was a sign that SOD activity was due to one of the copper SOD enzymes (SOD 1 or

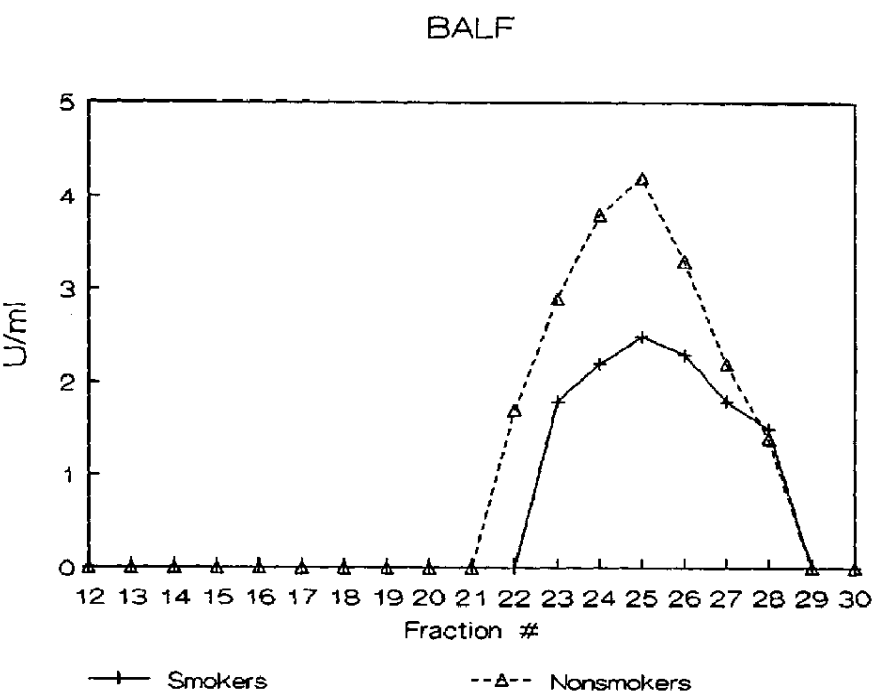

FIGURE 1. Gel filtration analysis of BALF SOD activity. Numbers on the X axis represent fraction number (3 mL/fraction) for column of C-150 Sephadex (1.5×75 cm) equilibrated in phosphate-buffered saline solution, pH 7.4, flown at a rate of 20 mL/h.

Table 1—*SOD Content by Category*

Category	No.	SOD, units/mg protein*
Control subjects	9	13±7[b]
Smokers	7	6±2[a]
Asthmatic subjects	12	17±10[b]

*Values are means±SD. Different superscripts connote statistically significant differences (ANOVA+LSD, $p<0.05$).

EC SOD).[1-6,8-10] Analysis of other BALF samples (1 smoker, 2 nonsmokers) showed that about 90% of each SOD activity was inhibited by antiSOD 1. Gel filtration analysis of pooled samples from 5 smokers or 5 control subjects yielded a single SOD activity peak (Fig 1). The peak came after that for ceruloplasmin (Fig 2), a protein with a molecular weight almost identical to EC SOD.[4-6,11] The BALF SOD peak eluted in the same fraction number as bovine liver SOD 1. The gel filtration data used for Figure 1 was derived from frozen samples. Therefore, an analysis was carried out on the same day as collection for three new individual samples (2 control subjects, 1 smoker). Each gave the same SOD activity peak fraction as for the frozen samples. Thus, inhibition and gel filtration data indicated that SOD 1 accounted for virtually all BALF SOD activity.

Discussion

This study indicated that BALF samples can be used to study human lung antioxidant enzyme activities. SOD activity was detectable in all samples studied, and values varied among individual subjects. Based on work with other biological fluids, EC SOD was expected to produce most of the BALF SOD activity. This enzyme accounts for most SOD activity in serum, lymph, spinal fluid, and synovial fluid.[4-6] In contrast, SOD 1 accounted for virtually all the detectable BALF SOD activity. This conclusion was based on gel filtration and antibody inhibition data. In the latter case, a small amount of activity (about 10%) persisted in the presence of antiSOD 1. This may have been noncatalytic superoxide scavenging by various proteins. This contention was supported by the presence of only one distinct SOD peak upon gel filtration. The cellular origin of BALF SOD 1 is uncertain.

BALF SOD activities for cigarette smokers and asthmatic subjects were compared to those for control subjects. Both smoking and asthma are thought to be associated with high degrees of radical-mediated oxidative stress.[1,2,7,12] Low SOD activities were found in a small number of smokers. Similarly, another recent study reports below-normal SOD activity in bronchoalveolar epithelial lining fluid

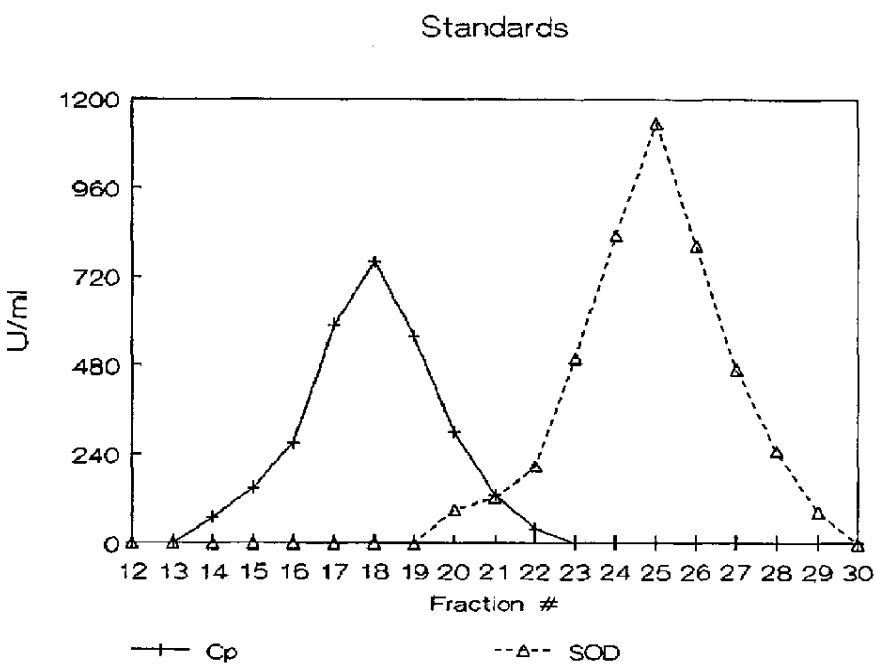

Figure 2. Gel filtration analysis of activity of purified ceruloplasmin (Cp) and copper-zinc SOD activity. Chromatography specifications were identical to those for Figure 1.

from a small number of smokers.[12] However, this other study assessed SOD activity by an assay which uses xanthine oxidase to generate superoxide substrate. Xanthine oxidase-based SOD assays can be inaccurate for certain biological fluids.[10] Thus, it is not known if this study used an appropriate assay for the samples examined. Nonetheless, the similar results for that study and the present one raise the possibility that smokers are prone to having low lung SOD activities. This may be due to inactivation of SOD 1 by reactions of smoke components with enzyme-bound copper. There is evidence that cigarette smoke inactivates other copper enzymes *in vivo*.[13,14] Chronic smoke exposure in hamsters restricts lung lysyl oxidase activities.[13] In addition, human cigarette smokers show low serum ceruloplasmin activity for the amount of ceruloplasmin protein.[14]

Several asthmatic subjects had relatively high BALF SOD activities, though there was no overall significant difference between control and asthmatic subjects. This large variation in BALF SOD for asthmatic subjects may indicate that more than one mechanism affects BALF SOD activities in such subjects. Asthmatic subjects may have a higher than normal need for SOD activity. Superoxide production from airspace cells removed from asthmatic subjects can be greater than when the cells are removed from control subjects.[7] However, superoxide production was found to be linked to disease activity. Since the asthmatic subjects in the present study had mild and stable asthma, their pulmonary oxidative stress may not have been excessive.

Conclusion

BALF contained detectable SOD 1 activity. Initial data suggested that activities may vary with different pulmonary stress states.

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